PCT/GB 97 / 02709
08 OCTOBER 1997
09/284009

The Patent Office

The Patent Office
Cardiff Road
Newport
Gwent
NP9 1RH

REC'D 12 NOV 1997
WIPO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation and Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

PRIORITY DOCUMENT

Signed

Dated 3 11 1997

Patents Form 1/77

Patents Act 1977
(Rule 16)

The Patent Office

30JAN97 E250120-1 002932
P01/7700 25.00

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Our reference

P1102

2. Patent application number
(The Patent Office will fill in this part)

30 JAN 1997

9701975.6

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

THE UNIVERSITY OF SHEFFIELD
WESTERN BANK
SHEFFIELD
SOUTH YORKSHIRE
S10 2TN

Patents ADP number *(if you know it)*

8912420002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

TARGETING OF STRESS INDUCIBLE GENES

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

WILLIAM JONES (YORK)
THE CRESCENT
54 BLOSSOM STREET
YORK
YO2 2AP

Patents ADP number *(if you know it)*

2253003

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing *(day / month / year)*

Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if*

a) *any applicant named in part 3 is not an inventor, or*
b) *there is an inventor who is not named as an applicant, or*
c) *any named applicant is a corporate body.*

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	22
Claim(s)	
Abstract	
Drawing(s)	

Rules

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature William Jones Date

WILLIAM JONES (YORK) 30.01.1997

12. Name and daytime telephone number of person to contact in the United Kingdom

JULIE M FYLES (Dr) 01904 610586

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

TARGETING OF STRESS INDUCIBLE GENES

The invention relates to a method of drug delivery; means therefor including components thereof which have particular, but not exclusive, application in cancer therapy development.

Macrophages often comprise 20-60% of the tumour cell mass in breast carcinomas and form intimate contacts with malignant cells. This has long been thought to represent part of the host's defence mechanisms against the tumour; however, their function at such sites in the body remains an enigma at present as macrophages isolated from human or murine tumours exhibit reduced tumouricidal, phagocytic and antigen-presenting activities compared to those from normal tissues (1).

Monocytes are produced in the bloodstream and extravasate (i.e. exit) into surrounding tissues including such diseased tissues as malignant tumours and atherosclerotic plaques, where they differentiate into macrophages and perform immune, secretory, phagocytic and other functions. Monocytes and macrophages are collectively termed mononuclear phagocytes. As tissue macrophages have a lifespan of 60 to 90 days and the number of macrophages in tumours remains constant, it is believed that there is a constant attachment of monocytes to the tumour endothelium and influx of monocytes into the tumour cell mass

Hypoxia, that is very low levels of oxygen, exist only in some forms of diseased tissue (e.g. malignant tumours, ischaemic heart tissue etc). Hypoxia

and/or hypoglycaemia is thought to occur in growing tumours when the increasing metabolic demands of the rapidly expanding tumour cell population outstrip the supply of oxygen/glucose etc., made available to them by simple diffusion across the tumour mass from vessels in surrounding normal tissues.

Ischaemia, that is, a deficiency of blood flow to part of the body also exists in some forms of diseased tissue (e.g. malignant tumours, ischaemic heart tissue etc). Malignant tumours tend to outgrow their blood supply and often have ischaemic areas of tumour cell apoptosis and necrosis. Moreover, the vascular supply in some tumour areas can collapse resulting in the formation of further ischaemic areas. Ischaemic tissues are also found in coronary artery disease, diabetic retinopathy and following strokes. Cells in such areas of vascular occlusion or collapse experience various forms of metabolic stress such as glucose deprivation, low pH, elevated levels of lactate and pyruvate, ion or osmatic imbalance and hypoxia.

Our recent and surprising data indicate that once monocytes enter a tumour from the bloodstream, they rapidly differentiate into macrophages and preferentially congregate in <u>hypoxic</u> (i.e. poorly vascularised and necrotic) sites deep within a tumour mass remote from blood vessels. Refer to Figure 1, which represents a bar chart of the Distribution of Macrophages in Relation to Blood Vessels. Moreover, breast tumours, with more hypoxic/necrotic areas, are more heavily infiltrated with macrophages, which preferentially locate to, or around, the necrotic sites (refer to Figure 2, which represents a bar chart of the Association of Macrophage Index with Necrosis in Breast Carcinomas). Experimental hypoxia has been shown to induce the production of angiogenic factors by macrophages *in vitro* (2). Taken together these data

could underpin our recent finding, that increased numbers of macrophages in breast tumours equate with higher levels of angiogenesis and increased fatalities in breast cancer (3).

We examined tumour biopsies from 100 breast cancer patients, and found that macrophage infiltration was strongly associated with reduced relapse-free interval and overall survival - even in lymph node negative patients alone (i.e. the better prognosis group). Indeed macrophage infiltration proved to be almost as important in predicting outcome as lymph node status in breast cancer. We went on to see if this was due to an effect of macrophages on such important parameters of tumour aggressiveness as the mitotic index, receptor status, degree of angiogenesis, etc. It was at this point that a highly significant and unexpected correlation between the degree of macrophage infiltration within a tumour mass and angiogenesis emerged, suggesting a role for this cell type in some or all of the steps of tumour angiogenesis (3).

Our unique observations suggest that, since the entry or presence of macrophages into or in such diseased tissues appears to be deleterious to the patient, therapies specifically focused on either blocking their entry, their destruction and/or their exploitation to carry therapeutic agents into such diseased tissues could prove to have therapeutic benefits.

Monoclonal antibodies have been considered for may years to be the best way of delivering cytotoxic agents to tumours, but this approach has so far been disappointing in clinical trials (4, 5). The main reason for the lack of

[redacted] studies with radiolabelled antibodies, typically only 0.001-0.01% of the injected dose

localises to each gram of solid tumours in humans (6, 7). The poor penetration of antibodies is thought to be due to a number of factors. Firstly, the antibodies must cross the physical barrier of the endothelial cell layer in tumour blood vessels and the, often dense, fibrous stroma packed between tumour cell areas. Secondly, the dense packing of tumour cells and tight junctions between epithelial tumour cells hinder the transport of the antibody within the tumour mass. Thirdly, the absence of lymphatics within the tumour contributes to the build up of a high interstitial pressure which opposes the influx of molecules into the tumour core.

One solution to the poor penetration of antibody conjugates into solid tumours would be to attack the endothelial cells in the tumour, instead of the tumour cell themselves, which are readily accessible to intravenously injected antibody. This then leads to destruction of the tumour blood vessels and the death of neighbouring tumour cells which rely on the blood supply for oxygen and nutrients. Early studies using murine models have met with some success in this area (8), but there remains the problem in humans of how to target tumour endothelial cells and not those in normal tissues. Furthermore, as mentioned earlier, our work strongly suggests the tumour necrosis resulting from this approach will trigger compensatory angiogenic activities in tumour-infiltrating macrophages in the vicinity. This would oppose the effects of the therapeutic agent.

Our inventive solution is to attach the agent (e.g. tumour or endothelial cell cytotoxin) to monocytes in the peripheral blood, that is the cells which gain entry to the tumour in large numbers to form tumour-infiltrating macrophages. Since monocytes swarm to the tumour site in large numbers as an early and ongoing event in tumour development, they can be used to carry therapeutic

agents (e.g. cytotoxic drugs or toxins) into the centre of solid tumours. In support of this suggestion is the fact that many studies have shown that malignant tumours actively recruit this cell type and that monocytes then follow a chemotactic trail produced by distressed (i.e. hypoxic/necrotic) tumour cells. Further, we have uniquely shown that mononuclear phagocytes congregate in tumour areas where they are most needed, i.e. hypoxic or stress areas, possibly to help initiate angiogenesis.

Although we have described the invention with particular reference to tumour cells, it can be used in any instance where mononuclear phagocytes infiltrate or are attracted to hypoxic tissue or conditions. Thus, the invention can be used during development to control the vascularisation of developing tissue, typically, but not exclusively, with a view to targeting a hypoxia regulatable agent so as to promote or enhance vascularisation. Alternatively the invention can be used to target hypoxia regulatable agents to damaged tissue, for example to tissue where de-vascularisation has occurred following damage to the vascular system via an amputation, stroke, cardiac arrest, extreme hypertension, ischaemia, burns etc.

It follows from the information provided herein that the invention may be used to prevent or reduce tissue vascularisation, or to promote or enhance vascularisation, or to simply deliver selected drugs to hypoxic sites where mononuclear phagocytes are typically present.

In addition, given our finding that mononuclear phagocytes infiltrate or are used with a view to targeting a stress regulatable agent such as an ischaemically regulatable agent. Thus, in the instance where mononuclear

phagocytes infiltrate a hypoxic site which is also ischaemic and thus characterised by glucose depravation, low pH, elevated levels of lactate or pyruvate, and/or hypoxia and it is possible to arrange for targeting of an ischaemically regulatable agent.

It is therefore an object of the invention to provide a novel drug delivery system which exploits the fact that mononuclear phagocytes collect or are attracted to hypoxic or stress sites.

It is yet a further object of the invention to provide a regulatable drug delivery system which enables the activation of said drug to be controlled, and more specifically, to be controlled so as to only be active under hypoxic and/or stress and/or ischaemic conditions.

It is yet a further object of the invention to provide a novel drug for use in the drug delivery system of the invention.

The invention, in its broadest aspect, comprises the use of mononuclear phagocytes to deliver therapeutic agents to tissues and especially hypoxic and/or stress and/or ischaemic sites.

According to a first aspect of the invention there is therefore provided a therapeutic composition comprising a hypoxia and/or stress regulatable agent and/or an agent that binds to a cell surface element of a mononuclear phagocyte.

It will therefore be apparent that the hypoxia and/or stress regulatable agent will be affected by hypoxic and/or stress conditions and typically affected so

as to only be active in such conditions. Moreover, said binding agent, which is typically coupled to said regulatable agent, attaches the composition to mononuclear phagocytes and so targets the regulatable agent, to sites typically infiltrated by mononuclear phagocytes. Thus in the instance where said mononuclear phagocytes penetrate hypoxic and/or stress sites said composition is suitably delivered to such sites and the regulatable agent becomes active.

The invention is elegant in so far as the body's own mechanisms are exploited for the specific delivery of drugs but the invention is safe in so far as the drugs remain inactive until exposed to hypoxic and/or stress conditions.

Given the above nature of the invention agents suitable for use in manufacturing the said composition will be known to those skilled in the art and therefore the following preferred embodiments are not intended to be exhaustive but rather illustrative.

For example, in one embodiment of the invention said hypoxia regulatable agent may comprise a therapeutic gene, that is to say a gene encoding a therapeutic agent which is under the control of a hypoxia sensitive agent such as a hypoxia regulated expression element i.e. a promoter or enhancer which is sensitive to hypoxia. Thus, under conditions of hypoxia said element will be activated so as to enable the gene encoding for the therapeutic agent to be expressed. In this embodiment of the invention the binding agent is optional, and indeed, may be substituted for an agent that ensures internalisation of

In addition, or alternatively, the hypoxia regulatable therapeutic gene may encode a prodrug activation enzyme, that is to say an enzyme which converts a relatively inactive drug into a more active one. An example of this kind of enzyme is thymidine phosphorylase which activates the 5-FU prodrugs capcetabine and furtulon. An other example of a pro-drug activation enzyme is the herpes simplex thymidine kinase or cytosine deaminase which, once internalised into the mononuclear phagocytes would act as a reservoir for activation of the prodrugs ganciclovir and 5-fluorocytosine.

Other examples of hypoxia regulatable therapeutic genes are to be found in PCT/GB95/00322.

Alternatively said hypoxia regulatable agent may comprise a bioreductase prodrug such as RSU 1069 which is activated at very low levels of oxygen as well as with contact with enzymes such as reductases. Thus, where not only hypoxia, but protein-protein interaction, typically enzymic, is required for activation of said regulatable agent, said therapeutic composition of the invention may further comprise an agent that activates said hypoxia regulatable agent, such as a reductase.

In a further embodiment of the invention said stress regulatable agent may comprise a therapeutic gene, that is a gene encoding a therapeutic agent which is under the control of an stress sensitive agent such as an stress regulated expression element i.e. a promoter or enhancer which is sensitive to any one or more of the factors characterising stress such as, for example, without limitation, glucose deprivation, low pH, elevated levels of lactate or pyruvate and/or ion or osmotic imbalance. Thus, under conditions of stress said elements will be activated so as to enable the gene encoding for the

therapeutic agent to be expressed. In this embodiment of the invention the binding agent is optional, and indeed, may be substituted for an agent that ensures internalisation of said therapeutic gene with a view to incorporating same into the mononuclear phagocyte genome.

As mentioned above, the stress regulatable therapeutic gene may encode a pro-drug activation enzyme as previously described.

Suitable examples of DNA sequences known to be activated by any of the aforementioned factors, or "stress factors" may be used in the invention and examples of such sequences include, without limitation, promoter and/or enhancer sequences activated by glucose deprivation, low pH or elevated levels of lactate or pyruvate and/or ion or osmotic imbalance. For example, the glucose-regulated proteins (grp's) such as grp78 and grp94 are highly conserved proteins known to be induced by glucose deprivation (19). The grp78 gene is expressed at low levels in most normal healthy tissues under the influence of basal level promoter elements, but has at least two critical "stress-inducible regulatory elements" upstream of the TATA element (19, 20). Attachment to a truncated, 632-base pair sequence of the 5'end of the grp78 promoter (which included these two stress-inducible regulatory sacs) confers high inducibility to glucose deprivation on reporter genes *in vitro* (20). Furthermore, use of this promoter sequence in a retroviral vector drove high level expression of a reporter gene in tumour cells in murine fibrosarcomas, particular in central, relatively ischaemic/necrotic sites (20). In addition it is thought that grp78 may be inducible by other stress factors (27).

those responsive to low pH. The genes for these proteins are known to have acid-inducible promoters, examples of which have been cloned and characterised in recent studies (21) indeed, it was demonstrated that the acid-inducibility of several such defined DNA sequences in hybrid DNA constructs consisting of each one of these fused to a reporter gene encoding a green fluorescent protein was sensitive to pH. Maximum induction was seen when the pH was dropped to 4.5.

Thus, said stress regulatable agent comprises a therapeutic gene under the control of an stress regulated expression element known to those skilled in the art. Notably, a stress regulated expression element is intended to include both homologous and heterologous elements i.e. that is to say promoters and/or enhancers for genes known to be expressed, or over-expressed, by mononuclear phagocytes in stress conditions; and promoters or enhancers for genes known to be over-expressed by other tissues in said conditions, respectively. Examples of the former group include the expression element of the osteopontin gene and thymidine phosphorylase gene. Other examples of homologous promoters or enhancers comprise those involved in the phagocytic activity of macrophages including CD36, CD68, thrombospondin, the $\alpha v\beta 3$ integin and low density lipoprotein receptors (25, 26).

Alternatively, or in addition, especially where internalisation of said therapeutic composition is required said therapeutic composition may further comprise an internalisation agent so as to ensure that the therapeutic composition is internalised by the mononuclear phagocytes. Agents which are suitable for ensuring internalisation of the therapeutic composition include, but are not limited to, plasminogen activation inhibitors (PAI-1 or PAI-2) or protease nexin (PN).

In yet a further preferred embodiment of the invention said binding agent is adapted to bind to any one or more cell surface mononuclear phagocyte molecules such as antigens or receptors. Further, said binding agent may comprise an antibody to any one or more of said molecules such as antigens or receptors, or an effective fragment of said antibody. Alternatively still said binding agent may comprise a suitable ligand either synthetically manufactured or naturally occurring.

A brief list of those cell surface molecules that may be targeted by said binding agent is as follows; the receptor for human Urokinase Plasminogen Activator (uPAR; CD87); the receptor for human Colony Stimulating Factor (CSF-1); CD63; CD64; CD11b; CR3; the scavenger receptor; all or part of the receptor for the various forms of human monocyte chemoattractant protein (MCP-1, 2, etc); CD14; mannose or mannose-6-phosphate surface receptors; CD16; or HLA-DR.

According to a yet further aspect of the invention there is provided a delivery system for targeting therapeutic compositions to hypoxic and/or stress sites comprising a hypoxia and/or stress regulatable agent and an agent for controlling the functional effectiveness thereof, and coupled thereto, a binding agent for a cell surface molecule of a mononuclear phagocyte.

According to a yet further aspect of the invention there is provided a method for targeting desired agents to hypoxic and/or stress sites comprising;

(ii) exposing said coupled agents to mononuclear phagocytes; and

(iii) allowing said mononuclear phagocytes to migrate, under conditions that support migration, either *in vitro, in vivo* or *ex vivo.*

According to a yet further aspect of the invention there is provided a method for treating conditions associated with hypoxic and/or stress states comprising administering to an individual to be treated the therapeutic composition of the invention.

According to a yet further aspect of the invention there is provided a method for treating conditions associated with hypoxic and/or stress states comprising withdrawing blood and/or serum from an individual to be treated and treating said blood and/or serum *in vitro* with a hypoxically and/or stress inducible therapeutic gene or fragment or part thereof under conditions that enable incorporation of said gene into the genome of mononuclear phagocytes and re-injecting said treated blood and/or serum into the individual either systemically or directly into a hypoxic and/or stress area.

According to a yet further aspect of the invention there is provided mononuclear phagocytes which have coupled thereto, or internalised therein, a hypoxia and/or stress regulatable agent and an agent that is adapted to bind to a mononuclear phagocyte ligand which is typically found on the cell surface of said mononuclear phagocyte.

According to a yet further aspect of the invention there is provided a method for selectively destroying a mononuclear phagocyte comprising attaching thereto or internalizing therein a cytotoxic, hypoxically and/or stress activated

agent and exposing said mononuclear phagocyte to hypoxic and/or stress conditions that occur either artificially by induction or occur/exist naturally.

In the instance where hypoxia and/or stress occurs/exists naturally said mononuclear phagocyte migrates in a normal manner to said hypoxic and/or stress area so that the said agent is only activated at a target area. In this way the potentially deleterious effects of mononuclear phagocytes in tumours is obviated. Moreover, having regard to the nature of said agent a bystander effect may be achieved, for example where said cytotoxic agent is released on death of said mononuclear phagocyte it may have a further deleterious effect on the hypoxic and/or stress tissue, such as, but not limited to, tumour tissue.

Many of the preferred embodiments hereinbefore described represent appropriate modifications of any one or more of the above referred to further aspects of the invention.

An embodiment of the invention will now be described by way of example only with reference to the following Table and figures wherein:

Table 1 represents specific examples of drug conjugates;

Materials and Method

Drug Delivery

[illegible] injection) into the general circulation so as to bind *in vivo* to the surface of

systemic mononuclear phagocytes and/or macrophages already resident in diseased tissues (e.g. malignant tumours). In support of the aforementioned mode of drug delivery is the finding that when a monoclonal antibody, specific for the Mac-1 antigen on macrophages, is conjugated to indium (a radioactive element) and injected intravenously into mice bearing a solid tumour, it was seen on scintographs to accumulate predominantly in the cancer lesion (9). Alternatively the drug conjugate can be exposed to monocytes *ex vivo,* following their purification from the blood of patients using such standard methods as Ficoll- Hypaque gradients and elutriation as described previously in (10).

Homing of blood monocytes loaded up with drug conjugates into malignant tumours can be augmented by prior treatment with conventional systemic therapies which induce local inflammation/necrosis in the diseased tissue (e.g. radiotherapy or chemotherapy in the case of cancer patients). This stimulates the release of chemoattractant factors for monocytes/macrophages such as MCP-1 (11, 12) and would thus enhance the delivery and hence the therapeutic efficacy of the drug conjugate at the diseased site.

Mode of production of selected drug conjugates

Drug 1. (with reference to Table 1) *RSU1069 - $F(ab)_2$ of a monoclonal antibody to CD87 (uPAR)*

This conjugate uses a highly specific $F(ab)_2$ fragment a monoclonal antibody to CD87 (urokinase plasminogen activator receptor; uPAR) to target naturally occurring uPAR on the surface of monocytes and macrophages.

A monoclonal antibody to CD87 is made as described in (13) and then cleaved/purified to a specific $F(ab)_2$ monoclonal antibody fragment using standard proteolytic methods. Depending upon the part of uPAR used to raise the antibody (i.e. as the antigen), the epitope for the antibody generated may either be in the (i) ligand (i.e. uPA) -binding portion of the uPAR (in which case the drug conjugate will only bind to unoccupied uPAR on monocytes/macrophages), or (ii) the non ligand (i.e. uPA) -binding portion of the uPAR (in which case the drug conjugate will bind to both unoccupied and unoccupied uPAR on monocytes/macrophages). The most effective drug uptake is likely to be achieved using the latter form of conjugate.

The fragment of the CD87 monoclonal antibody is conjugated to the bioreducative prodrug, RSU1069, by the latter being reacted with an agent such as arylazide to add a N-hydroxysuccinimide group. This is then cross-linked at neutral pH to the CD87 antibody fragment via amine groups to form a conjugate. This method is well established for conjugating drugs to proteins and is described fully in (14).

Drug 2. (with reference to Table 1) *i.e. RSU1069 - PAI-2*

This conjugate uses the affinity of plasminogen activator inhibitor 2 (PAI-2) for urokinase plasminogen activator receptor (uPAR) - urokinase plasminogen activator complexes to target the bioreductive prodrug to the surface of monocytes and macrophages. PAI-2 triggers the internalization of uPAR-uPA complexes, so the internalization by these cells of the bioreductive

[illegible]

Naturally occurring PAI-2 is obtained from the culture supernatant of human

blood monocytes stimulated maximally with interleukin 1 or 2 as described in (15). This is then purified to homogeneity in the usual manner by elution from an anti-PAI-2 immunoaffinity column. Alternatively, PAI-2 can be produced in a recombinant expression system and purified according to the method of (16). The PAI-2 preparation is then conjugated to RSU1069 using the method outlined as hereinbefore described for drug 1(14).

Drug 6. (with reference to Table 1) i.e. Interleukin-2 (IL-2) gene linked to hypoxia responsive promoter.

This gene is transferred to monocytes/macrophages using a replication-defective adenoviral vector. Efficient transfer of genes into human macrophages has been achieved with this method with expression of the gene in 40-80% of the cells exposed to the vector and lasting up to 3 weeks after gene transfer (10).

Defective retroviral vectors, direct DNA internalization or such non-gene viral gene transfer systems can also be used such as cationic lipids, liposomes, lectins or polymers. Genes other than IL-2 which could be of therapeutic benefit include such other immunomodlators as TNFα, or interferon gamma, prodrug activating enzymes, enzyme inhibitors, tumour antigens (to provoke the hosts immune reactivity to the tumour) and anti-oncogenes (antibodies or antisense RNA). It will be understood by those skilled in the art that the DNA construct used may, embody a number of these genes rather than just one and is not intended to limit the scope of the application.

The hypoxically inducible expression control sequence (promoter) for the Epo or PGK genes (or multiple copies thereof) is/are coupled to one or more of

the gene sequences of choice (e.g. IL-2 gene sequence) as described by one of us previously in (17).

Ex vivo gene transfer:

The method outlined in (10) involves incubating (under sterile conditions) freshly isolated blood monocytes or monocyte-derived macrophages (monocytes incubated overnight in teflon bags or on plastic cultured wells) in the presence of 100 plaque-forming units per cell of the purified replication-defective vector, Ad.RSVβ, harbouring the hypoxia-responsive promoter-IL-2 gene construct in RPM1 incubation medium (1ml/10^6 cells). Gene transfer may be enhanced by simultaneous treatment of the cells with 100U/ml of human interferon gamma. Cells are then washed to remove free viral particles and interferon gamma and reincubated at 37°C in fresh RPMI medium in teflon bags. The same adenoviral vector but harbouring the E.coli β-galactosidase gene (Ad.RSV.βgal) instead of the hypoxia-responsive promoter-IL-2 gene construct is used as a reporter gene (i.e. to check the efficiency of this gene transfer method to monocytes/macrophages *ex vivo*). The presence of the β-gal enzyme in cells after infection with Ad.RSV.βgal is then assessed using histochemical methods as described in (17). The transfected cells (10^8 to 10^9 cells) are then injected sterile back into the bloodstream or directly into the appropriate diseased tissue (e.g. malignant tumour) of the donor as in (10).

In vivo gene transfer:

[illegible] method [illegible] gene delivery described in (18). This involves injecting the vector (i.e. Ad.RSVβ) bearing

the hypoxia-responsive promoter-IL-2 gene construct into the bloodstream (primarily to label monocytes) and/or into the diseased tissue (at 10^9 to 10^7 plaque-forming units) to label tissue macrophages.

Results

The results are represented as exemplary drug conjugate candidates in Table 1. It will be understood by those skilled in the art that such conjugates represent selected examples and are not intended to limit the scope of the invention, furthermore it will be understood that indeed any one example of a candidate Part I drug conjugate may be used in conjunction with any one example of a Part II candidate and/or any example of a Part III candidate. Additionally it will be understood that any example of a Part III candidate may be used in conjunction with any example of a Part II and/or Part I candidate drug conjugate.

The invention hereinbefore described therefore represents a most elegant and effective means and method of delivering a therapeutic and/or cytotoxic agent to a hypoxic site by use of monocytes and/or macrophages and their natural ability to congregate at a hypoxic site.

TABLE 1 - SPECIFIC EXAMPLES OF DRUG CONJUGATES

	Part 1	Part II	Part III
Drug 1	*RSU1069*	*F(ab)2 to CD87 (uPAR)*	-
Drug 2	*RSU1069*	*PAI-2* (binds to receptor bound urokinase plasminogen activator (uPAR) & ensures internalization of drug)	-
Drug 3	*RSU1069*	*PAI-2* (binds to receptor bound uPAR & ensures internalization of drug)	*Cyt. P450* (reductase for activation of RSU1069 under hypoxia)
Drug 4	*RSU1069*	*F(ab)2 to CSF-1 receptor* (binds to human CSF receptor on surface of monocyte/ macrophages)	*Cyt. P450*
Drug 5	*Cytosine deaminase* enzyme	*F(ab)2 to CD63*	-

Drug 6	*Gene for Interleukin 2 (immunostimulatory cytokine) in Ad.RSV*	*Hypoxia-resp. promoter sequence*
Drug 7	*DNA sequence for soluble domain of VEGF receptor (eg. flk-1) in Ad. RSV*	*Hypoxia-resp. promoter sequence*

REFERENCES

1. O'Sullivan C and Lewis CE (1994) J. Pathol. 172: 229-235.
2. Knighton DR, et al. (1983) Science. 221: 1238-85
3. Leek RD, Lewis CE, Whitehouse R and Harris AL. Association of macrophages with angiogenesis and prognosis in invasive breast cancer. (Cancer Research in press).
4. Vaickus L and Foon KA. (1991). Cancer Invest. 9:195-209.
5. Byers VS and Baldwin RW. (1988). *Immunol.* 65: 329-409
6. Sands H. (1988). *Antibody Immunoconjug. Radiopharm* 1:213-226.31.
7. Epenetos AA. et al. (1986). *Cancer Res.* 46:3183-91.
8. Derbyshire EJ and Thorpe PE. (1996). In *"Tumour Angiogenesis"* Eds R. Bicknell, CE Lewis & N. Ferrara. Oxford University Press, Oxford. (in press).
9. Collette B. et al. (1988). *Cancer Immunol. Immuno. Ther.* 26: 237-242.
10. Haddada H,, et al. (1993). *Biochem. Biophys. Res. Commun.* 195: 1174-83.
11. Mantovani A., et al. (1993). *Res. Immunol.* 144: 280-83.
12. Martinet N., et al. (1992). *Cancer.* 70: 854-60.
13. Ronne E., et al. (1991). *FEBS Letts.* 288: 233-36.
14. Gabor F., et al. (1995). *Archiv. der Pharmazie.* 328: 775-80.
15. Gyetko M. R., et al. (1993). *J. Leuk. Biol.* 53: 598-601.
16. Steven F., et al. (1991). *Eur. J. Biochem.* 196: 431-8.
17. Ratcliffe P. J., Firth J. D., and Harris A. L. (1994). Patent application

Attenello J. W., Lee A.S., (1984). *Science* 226:187 1909.

20. Gazit G., Kane S. E., Nichols P., Lee A.S., (1995). *Cancer Res.* 55: 1660-63.

21. Valdivia R. H., Falkow S., (1996). *Molecular Microbiology* 22: 367-78.

22. Brown L.F., Papadopoulossergiou A., Berse B., Manseau E. J., Tognazzi K., Perruzzi C. A., Dvorak H. F., Senger D. R., (1994). *AM. J. Pathol.* 145: 610-623.

23. Hijiya N., Setoguchi M., Matsura K., Higuchi Y., Akizuki S., Yamamoto S., (1994). *Biochem. J.* 303: 255-262.

24. Takahashi Y., Bucana C. D., Liu W. B., Yoneda J., Kitadi Y., Cleary K. R., Ellis L. M., (1996). *J. Natl. Canc. Inst.* 88: 1146-1151.

25. Griffiths L., Dachs GU., Bicknell R., Harris A. L., & Stratford I., (1997). *Cancer Res.* 57 (in press; Feb. issue).

26. Savill J. (1996). *Biochem. Soc. Trans.* 24: 1065-69.

27. Sugawara S., Takeda K., Lee A., Dennert G., (1993). *Cancer Res.* 53: 6001-5005.

① 97/02709

② 28 to 9

③ William Jones (York)